

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6: C12Q 1/68	A1	(11) International Publication Number: WO 99/50452 (43) International Publication Date: 7 October 1999 (07.10.99)
(21) International Application Number: PCT/IB99/00848 (22) International Filing Date: 16 March 1999 (16.03.99) (30) Priority Data: 60/079,651 27 March 1998 (27.03.98) US		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
(71) Applicant: JOHNSON & JOHNSON RESEARCH PTY. LIMITED (AU/AU); 15 Blue Street, North Sydney, NSW 2060 (AU). (72) Inventors: TODD, Alison, V.; 10 Concill Place, Glebe, NSW 2037 (AU). FUERY, Caroline, J.; 59 Bradley Drive, Carlingford, Sydney, NSW 2118 (AU). CAIRNS, Murray, J.; 21 Terry Avenue, Woy Woy, NSW 2256 (AU).		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: CATALYTIC NUCLEIC ACID-BASED DIAGNOSTIC METHODS		
(57) Abstract		
<p>This invention provides methods and kits useful for determining whether a subject is afflicted with a disorder characterized by the presence of one or more known nucleic acid mutations. The instant methods comprise steps of nucleic acid molecule isolation, amplification, contact with one or more catalytic nucleic acid molecules specifically cleaving a target sequence present either in the case of disorder or wild-type, but not both, and determining cleavage of the amplified segment(s).</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	HU	Hungary	TR	Turkey
BG	Bulgaria	IE	Ireland	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IL	Israel	MN	Mongolia	UA	Ukraine
BK	Brazil	IS	Iceland	MR	Mauritania	UG	Uganda
BY	Belarus	IT	Italy	MW	Malawi	US	United States of America
CA	Canada	JP	Japan	NB	Niger	UZ	Uzbekistan
CF	Central African Republic	KE	Kenya	NL	Netherlands	VN	Viet Nam
CG	Congo	KG	Kyrgyzstan	NO	Norway	YU	Yugoslavia
CH	Switzerland	KP	Democratic People's Republic of Korea	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KR	Republic of Korea	PL	Poland		
CM	Cameroon	KZ	Kazakhstan	PT	Portugal		
CN	China	LC	Saint Lucia	RO	Romania		
CU	Cuba	LI	Liechtenstein	RU	Russian Federation		
CZ	Czech Republic	LK	Sri Lanka	SD	Sudan		
DE	Germany	LR	Liberia	SE	Sweden		
DK	Denmark			SG	Singapore		
EE	Estonia						

CATALYTIC NUCLEIC ACID-BASED DIAGNOSTIC METHODS

5 Throughout this application, various publications are cited. The disclosure of these publications is hereby incorporated by reference into this application to describe more fully the state of the art to which this invention pertains.

10

Field of the Invention

This invention relates to methods of diagnosing disorders characterized by known nucleic acid mutations.

15 The instant methods employ the use of catalytic nucleic acid molecules, and are useful in connection with diagnosing such disorders as cancer and AIDS.

Background of the Invention

20

A variety of inherited and acquired diseases are associated with genetic variations such as point mutations, deletions and insertions. Some of these variations are directly associated with the presence of disease, while others correlate with disease risk and/or prognosis. There are more than 500 human genetic diseases which result from mutations in single genes (21, 22). These include cystic fibrosis, muscular dystrophy, $\alpha 1$ -antitrypsin deficiency, phenylketonuria, sickle cell anemia or trait, and various other hemoglobinopathies (21, 22). Furthermore, individuals with increased susceptibility to several common polygenic conditions, such as atherosclerotic heart disease, have been shown to have an association with the inheritance of particular DNA sequence polymorphisms.

Cancer is thought to develop due to the accumulation of genetic lesions in genes involved in cellular proliferation or differentiation. The ras proto-oncogenes, K-ras, N-ras and H-ras, and the p53 tumor suppressor gene are examples of genes which are frequently mutated in human cancers. Specific mutations in these genes leads to an increase in transforming potential. Genetic analysis would be invaluable in the clinic for assessing disease risk, diagnosis of disease, predicting a patient's prognosis or response to therapy, and monitoring a patient's progress. The introduction of such genetic tests, however, will depend on the development of simple, inexpensive, and rapid assays for genetic variations.

15

Methods of *in vitro* nucleic acid amplification have wide-spread applications in genetics and disease diagnosis. In the last decade many techniques for amplification of nucleic acid have been described. These include the polymerase chain reaction (PCR) (1-7), the ligase chain reaction (LCR) (8), the strand displacement amplification assay (SDA) (9) and transcription-mediated amplification (TMA) (10, 11) (also known as self-sustained sequence replication (SSR)). The amplification products (amplicons) produced by PCR, LCR and SDA are DNA, whereas RNA amplicons are produced by TMA. DNA or RNA templates, generated by these protocols or others, can be analyzed for the presence of sequence variation (i.e. mutation) associated with the disease to be ascertained.

As with nucleic acid amplification, catalytic nucleic acids have been studied intensively in recent years. The potential for suppression of gene function using catalytic nucleic acids as therapeutic agents is widely discussed in the literature (12-18). Catalytic

RNA molecules (ribozymes) have been shown to be capable of cleaving both RNA (12) and DNA (17) molecules.

Similarly, catalytic DNA molecules (DNAzymes) have also been shown to be capable of cleaving both RNA (13, 19)

5 and DNA (18) molecules. Catalytic nucleic acid can only cleave a target nucleic acid sequence, provided that target sequence meets minimum sequence requirements.

The target sequence must be complementary to the hybridizing regions of the catalytic nucleic acid and

10 the target must contain a specific sequence at the site of cleavage. Examples of such sequence requirements at the cleavage site include the requirement for a purine:pyrimidine sequence for a class of DNAzyme cleavage (10-23 model) (19), and the requirement for the

15 sequence uridine:H where H can equal A, C or U but not G, for the hammerhead ribozymes (23).

In addition to their therapeutic potential, catalytic nucleic acid molecules can also distinguish

20 between targets which differ by a single point mutation (14-16). This is achieved by targeting a specific sequence which is present in wild-type but not mutant templates or vice versa. So far, this capacity for discrimination has only been exploited as a method for

25 therapeutic manipulation of gene expression.

A review by Nollau-Wagener (24) compared several methodologies for the detection of point mutations with respect to the type of nucleic acid analyzed, the

30 percentage of mutations detected, the time and cost of performing the assay, and problems relating to the use of toxic reagents. Each of the methodologies examined had its drawbacks. For example, denaturing gradient gel electrophoresis is time consuming, RNAase A cleavage can

35 only detect about 70% of possible mutations, and chemical cleavage involves the use of toxic substances.

Another method, known as restriction fragment length polymorphism (RFLP), involves ascertaining whether a restriction enzyme site is present or absent 5 at the locus of interest. In rare instances, mutations can be detected because they happen to lie within a naturally occurring restriction endonuclease recognition/cleavage site (31).

- 10 The inclusion of mismatched bases within primers used to facilitate *in vitro* amplification can result in the induction of artificial restriction endonuclease recognition/cleavage sites, and hence an increase in the number of loci which can be analyzed by RFLP (32).
15 Modified primers containing mismatched bases have been used to induce artificial recognition/cleavage sites for restriction endonucleases at critical codons within the *ras* gene family (33-35). The general rules for designing primers which contain mismatched bases located
20 near the 3' termini of primers have been established (36).

- Although the use of mismatched primers has expanded the utility of RFLP analysis, the technique is still
25 limited by the fact that a minimum of four base pairs is required for recognition and cleavage by a restriction enzyme.

Summary of the Invention

This invention provides a method of determining whether a subject is afflicted with a disorder

5. characterized by the presence of a known nucleic acid mutation, which comprises the steps of (a) isolating a sample of nucleic acid molecules from the subject; (b) (i) amplifying the nucleic acid segment present in the isolated sample, which segment is known to contain the
10. mutation in a subject afflicted with the disorder, and (ii) under suitable conditions, contacting the resulting amplified segment with a catalytic nucleic acid molecule which specifically recognizes and cleaves a target sequence present either (1) in the nucleic acid segment
15. having the known mutation or (2) in the corresponding wild-type nucleic acid segment, but not both, with the proviso that step (ii) can be performed either subsequent to or concurrently with step (i); and (c) determining whether the catalytic nucleic acid molecule in step
20. (b) (ii) cleaves the amplified segment, so as to determine whether the subject is afflicted with the disorder.

This invention also provides a method of determining whether a subject is afflicted with a disorder

25. characterized by the presence of a plurality of known nucleic acid mutations, which comprises the steps of (a) isolating a sample of nucleic acid molecules from the subject; (b) (i) amplifying the nucleic acid segment present in the isolated sample, which segment is known to contain the plurality of mutations in a subject afflicted with the disorder, and (ii) under suitable conditions, contacting the resulting amplified segment with a plurality of catalytic nucleic acid molecules, each of which specifically recognizes and cleaves a target
30. sequence present either (1) in the nucleic acid segment having the known mutation or (2) in the corresponding
35. sequence present either (1) in the nucleic acid segment having the known mutation or (2) in the corresponding

wild-type nucleic acid segment, but not both, with the proviso that step (ii) can be performed either subsequent to or concurrently with step (i); and (c) determining whether each of the catalytic nucleic acid molecules in 5 step (b)(ii) cleaves the amplified segment, so as to determine whether the subject is afflicted with the disorder.

This invention further provides a method of 10 determining whether a subject is afflicted with a disorder characterized by the presence of a plurality of known nucleic acid mutations, which comprises the steps of (a) isolating a sample of nucleic acid molecules from the subject; (b) (i) amplifying the nucleic acid segments 15 present in the isolated sample, which segments collectively are known to contain the plurality of mutations in a subject afflicted with the disorder, and (ii) under suitable conditions, contacting the resulting amplified segments with a plurality of catalytic nucleic acid molecules, each of which specifically recognizes and 20 cleaves a target sequence present either (1) in one of the nucleic acid segments having one of the known mutations or (2) in the corresponding wild-type nucleic acid segment, but not both, with the proviso that step (ii) can be 25 performed either subsequent to or concurrently with step (i); and (c) determining whether each of the catalytic nucleic acid molecules in step (b)(ii) cleaves the amplified segment containing its respective target sequence, so as to determine whether the subject is 30 afflicted with the disorder.

Finally, this invention provides kits for use in practicing the instant diagnostic methods. The first instant kit comprises (a) a catalytic nucleic acid 35 molecule which specifically recognizes and cleaves a target sequence present either (i) in a nucleic acid

segment having a mutation known to be characteristic of a disorder or (ii) in the corresponding wild-type nucleic acid segment, but not both, and (b) a nucleic acid reagent suitable for use in amplifying the nucleic acid 5 segment containing the target sequence.

The second instant kit comprises (a) a 10-23 DNAzyme which specifically recognizes and cleaves a target sequence present either (i) in a nucleic acid 10 segment having a mutation known to be characteristic of a disorder or (ii) in the corresponding wild-type nucleic acid segment, but not both, and (b) a DNA primer suitable for initiating amplification of the segment under polymerase chain reaction conditions, which primer 15 contains at least one purine ribonucleotide residue which serves as the 5' side of the site within the amplified segment recognized and cleaved by the 10-23 DNAzyme.

20 The third instant kit comprises (a) a first DNA primer which comprises a zymogene encoding a 10-23 DNAzyme that specifically recognizes and cleaves a target sequence present either (i) in a nucleic acid segment having a mutation known to be characteristic of 25 a disorder or (ii) in the corresponding wild-type nucleic acid segment, but not both, which first primer is suitable for initiating amplification of the segment under polymerase chain reaction conditions; and (b) a second DNA primer suitable for initiating amplification 30 of the segment under polymerase chain reaction conditions, which second primer contains at least one purine ribonucleotide residue which serves as the 5' side of the site within the amplified segment recognized and cleaved by the 10-23 DNAzyme, such that, upon

amplification, (i) the resulting amplified nucleic acid molecule comprises the 10-23 DNAzyme, and (ii) the amplified nucleic acid segment is recognized and cleaved *in cis* by the DNAzyme.

Detailed Description of the Invention

This invention provides methods employing catalytic nucleic acids to determine whether a subject is afflicted 5 with a disorder characterized by the presence of one or more known nucleic acid mutations. These methods are collectively applicable to scenarios where the disorder is characterized by (i) a single mutation within a single nucleic acid segment, or (ii) a plurality of mutations 10 within a single nucleic acid segment, or (iii) a plurality of mutations within a plurality of nucleic acid segments. For each mutation tested for by nucleic acid amplification, specific cleavage, and analysis, the instant methods provide a "yes or no" answer as to whether 15 the mutation exists. This answer in turn ultimately leads to a "yes or no" answer as to whether the corresponding disorder is present in the subject.

Specifically, this invention provides a method of 20 determining whether a subject is afflicted with a disorder characterized by the presence of a known nucleic acid mutation, which comprises the steps of (a) isolating a sample of nucleic acid molecules from the subject; (b) (i) amplifying the nucleic acid segment present in the 25 isolated sample, which segment is known to contain the mutation in a subject afflicted with the disorder, and (ii) under suitable conditions, contacting the resulting amplified segment with a catalytic nucleic acid molecule which specifically recognizes and cleaves a target sequence present either (1) in the nucleic acid segment 30 having the known mutation or (2) in the corresponding wild-type nucleic acid segment, but not both, with the proviso that step (ii) can be performed either subsequent to or concurrently with step (i); and (c) determining 35 whether the catalytic nucleic acid molecule in step

(b) (ii) cleaves the amplified segment, so as to determine whether the subject is afflicted with the disorder.

This invention also provides a method of determining
5 whether a subject is afflicted with a disorder characterized by the presence of a plurality of known nucleic acid mutations, which comprises the steps of (a) isolating a sample of nucleic acid molecules from the subject; (b) (i) amplifying the nucleic acid segment
10 present in the isolated sample, which segment is known to contain the plurality of mutations in a subject afflicted with the disorder, and (ii) under suitable conditions, contacting the resulting amplified segment with a plurality of catalytic nucleic acid molecules, each of
15 which specifically recognizes and cleaves a target sequence present either (1) in the nucleic acid segment having the known mutation or (2) in the corresponding wild-type nucleic acid segment, but not both, with the proviso that step (ii) can be performed either subsequent
20 to or concurrently with step (i); and (c) determining whether each of the catalytic nucleic acid molecules in step (b)(ii) cleaves the amplified segment, so as to determine whether the subject is afflicted with the disorder.

25

This invention further provides a method of determining whether a subject is afflicted with a disorder characterized by the presence of a plurality of known nucleic acid mutations, which comprises the steps of (a)
30 isolating a sample of nucleic acid molecules from the subject; (b) (i) amplifying the nucleic acid segments present in the isolated sample, which segments collectively are known to contain the plurality of mutations in a subject afflicted with the disorder, and
35 (ii) under suitable conditions, contacting the resulting amplified segments with a plurality of catalytic nucleic

acid molecules, each of which specifically recognizes and cleaves a target sequence present either (1) in one of the nucleic acid segments having one of the known mutations or (2) in the corresponding wild-type nucleic acid segment,

5 but not both, with the proviso that step (ii) can be performed either subsequent to or concurrently with step (i); and (c) determining whether each of the catalytic nucleic acid molecules in step (b)(ii) cleaves the amplified segment containing its respective target

10 sequence, so as to determine whether the subject is afflicted with the disorder.

The instant methods can be used to diagnose disorders in any subject. As used herein, "subject" means any animal, including, for example, mice, rats, dogs, guinea pigs, ferrets, rabbits, and primates. In the preferred embodiment, the subject is a human.

The disorder diagnosed by the instant invention can

20 be any disorder characterized by the presence of at least one known nucleic acid mutation, which mutation is missing when such disorder is absent. Such disorders are well known in the art and include, by way of example, cancer, AIDS, cystic fibrosis, muscular dystrophy, α1-anti-

25 trypsin deficiency, phenylketonuria, sickle cell anemia or trait, and various other hemoglobinopathies. In one embodiment, the disorder is selected from the group consisting of cancer, AIDS, and cystic fibrosis. In the preferred embodiment, the disorder is cancer. In the

30 Experimental Details section which follows, numerous examples are given of specific mutations, target sequences containing same, and catalytic nucleic acids used for diagnosing such disorders as cancer, AIDS and cystic fibrosis.

As used herein, "catalytic nucleic acid molecule" means a DNA molecule (also known in the art as a "DNAzyme") or RNA molecule (also known in the art as a "ribozyme") which specifically recognizes and cleaves a 5 distinct target nucleic acid sequence. For both DNAzymes and ribozymes, the target nucleic acid sequence can be either DNA or RNA.

The nucleic acid sequence in which the known 10 disorder-characterizing mutation(s) resides (i.e., the sequence amplified in the instant methods) can be a DNA or RNA sequence. These mutation(s) include, for example, point mutations, deletion mutations, insertion mutations and frame-shift mutations. Each of the amplified nucleic 15 acid segment and catalytic nucleic acid molecule can be either DNA or RNA. In one embodiment, the amplified nucleic acid segment is RNA and the catalytic nucleic acid molecule is either DNA or RNA. In a further embodiment, the amplified nucleic acid segment is DNA and the 20 catalytic nucleic acid molecule is either RNA or DNA (25).

Methods for isolating and amplifying nucleic acid molecules used in the instant invention are well known in the art. More specifically, methods of isolating a sample 25 of nucleic acid molecules from the subject include, for example, phenol chloroform extraction, quick lysis, capture on columns and polymer capture (20, 26-29). Methods of amplifying a nucleic acid sequence include, for example, PCR, LCR, SDA and TMA (also known as (SSR)) (1- 30 11).

Suitable conditions for contacting an amplified nucleic acid segment containing a target sequence with a catalytic nucleic acid molecule so as to permit specific 35 recognition and cleavage of the target sequence are well

known in the art. In addition, such conditions are exemplified in the Experimental Details section below.

Methods of determining whether a catalytic nucleic acid molecule cleaves an amplified nucleic acid segment are also routine in the art. Such methods include, by way of example, polyacrylamide gel electrophoresis and capillary electrophoresis (20, 30).

10 In the preferred embodiment of this invention, (a) the amplification is performed using a polymerase chain reaction; (b) the catalytic nucleic acid molecule is a 10-23 DNAzyme; and (c) the polymerase chain reaction employs a DNA primer (i.e., a "chimeric" primer) 15 suitable for initiating amplification of the segment, which primer contains at least one purine ribonucleotide residue which serves as the 5' side of the site within the amplified segment recognized and cleaved by the 10-23 DNAzyme. This purine ribonucleotide residue in the 20 chimeric primer is required for cleavage by the 10-23 DNAzyme. Thus, using this chimeric primer permits the 10-23 DNAzyme cleavage site to be generated in a PCR reaction. The chimeric primer can also include, for 25 example, a ribonucleotide residue that serves as the 3' side of the site recognized and cleaved by the 10-23 DNAzyme.

In one form of this embodiment, the amplified segment is recognized and cleaved *in trans* by the DNAzyme. In 30 another form, (a) the polymerase chain reaction employs a second DNA primer suitable for initiating amplification of the segment, which second primer comprises a zymogene encoding a 10-23 DNAzyme such that, upon amplification, the resulting amplified nucleic acid molecule comprises 35 the 10-23 DNAzyme; and (b) the amplified nucleic acid segment is recognized and cleaved *in cis* by the DNAzyme.

As used herein, "cis" cleavage by a DNAzyme shall mean that the DNAzyme recognizes and cleaves a sequence coexisting therewith on the same amplified nucleic acid molecule. Trans cleavage shall mean that the DNAzyme cleaves a substrate located on a different molecule. Finally, "zymogene" shall mean a nucleic acid sequence which comprises the anti-sense (i.e. complementary) sequence of a catalytic nucleic acid molecule, and whose transcription product is the catalytic nucleic acid molecule itself.

This invention still further provides kits for use in practicing the instant diagnostic methods. The first instant kit comprises (a) a catalytic nucleic acid molecule which specifically recognizes and cleaves a target sequence present either (i) in a nucleic acid segment having a mutation known to be characteristic of a disorder or (ii) in the corresponding wild-type nucleic acid segment, but not both, and (b) a nucleic acid reagent suitable for use in amplifying the nucleic acid segment containing the target sequence.

In one embodiment, the kit comprises a plurality of catalytic nucleic acid molecules. The nucleic acid reagent suitable for use in amplifying the nucleic acid segment containing the target sequence can be, for example, a nucleic acid primer. In one embodiment, the kit comprises a plurality of such nucleic acid reagents.

More specifically, the second instant kit comprises (a) a 10-23 DNAzyme which specifically recognizes and cleaves a target sequence present either (i) in a nucleic acid segment having a mutation known to be characteristic of a disorder or (ii) in the corresponding wild-type nucleic acid segment, but not both, and (b) a DNA primer

suitable for initiating amplification of the segment under polymerase chain reaction conditions, which primer contains at least one purine ribonucleotide residue which serves as the 5' side of the site within the 5 amplified segment recognized and cleaved by the 10-23 DNAzyme.

The third instant kit comprises (a) a first DNA primer which comprises a zymogene encoding a 10-23 10 DNAzyme that specifically recognizes and cleaves a target sequence present either (i) in a nucleic acid segment having a mutation known to be characteristic of a disorder or (ii) in the corresponding wild-type nucleic acid segment, but not both, which first primer 15 is suitable for initiating amplification of the segment under polymerase chain reaction conditions; and (b) a second DNA primer suitable for initiating amplification of the segment under polymerase chain reaction conditions, which second primer contains at least one 20 purine ribonucleotide residue which serves as the 5' side of the site within the amplified segment recognized and cleaved by the 10-23 DNAzyme, such that, upon amplification, (i) the resulting amplified nucleic acid molecule comprises the 10-23 DNAzyme, and (ii) the 25 amplified nucleic acid segment is recognized and cleaved in cis by the DNAzyme.

In one embodiment, the instant kits further comprise one or more of the following: (a) reagents 30 useful for isolating a sample of nucleic acid molecules from a subject being diagnosed; (b) reagents useful for amplifying a nucleic acid segment present in the isolated sample, which segment is known to contain a mutation in a subject afflicted with the disorder; and (c) reagents 35 useful for creating suitable reaction conditions for catalytic nucleic acid activity. The reagents in

components (a)-(c) of the instant kits can either be obtained commercially or made according to well known methods in the art, as exemplified in the Experimental Details section below.

5

The components of the instant kits can be in solution or lyophilized as appropriate. In one embodiment, the components of the instant kits are in the same compartment, and in another embodiment, the 10 components of the instant kit are in separate compartments. In the preferred embodiment, the kits further comprise instructions for use.

This invention will be better understood by reference 15 to the Experimental Details which follow, but those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative of the invention as described more fully in the claims which follow thereafter.

Experimental Details

The examples of DNAzymes and ribozymes listed below
5 are based on a 10-23 DNAzyme (19) and are designed to cleave the following medically important targets. The examples of ribozymes listed below are based on the hammerhead ribozyme (12).

10 I. DNAzymes

where R = purine, either A or G.
 Y = pyrimidine, either C, T or U.
 H = A, T, U, or C (not G).
 D = A, T, U, or G (not C).
 B = C, T, U, or G (not A).
 V = A, C, or G (not T, not U).
 W = T, U, or A.

italics = bases which are artificially introduced by *in vitro* amplification using primers containing bases which are mismatched with respect to the target sequence.

bold = target base or sequence for analysis.

underlined = recognition site (RY/R).

E = modified sequence (primer-induced artificial sequence).

30 A. Acquired Disease

(1) Cancer

(a) K-ras codon 12, position 2 - mutant (G to C, U or A)

35 5' - GUU GGA GCU GGU GGC GUA GGC - 3' wildtype
 RNA
 5' - GUU GGA GCU GYU GGC GUA GGC - 3' mutant RNA
 40 3' - CAA CCT CGA RA CCG CAT CCG - 5' DNAzyme
 A G
 G G
 C C
 A T
 A A
 C G
 A C
 T

5' - GUU GGA GCU GAU GGC GUA GGC - 3' mutant RNA
 3' - CAA CCU CGA CUA CCG CAU CCG - 5' antisense

5 5' - GCC UAC GCC AUC AGC UCC AAC - 3' antisense
 3' - CGG ATG CGG AG TCG AGG TTG - 5' DNAzyme

	A	G
	G	G
10	C	C
	A	T
	A	A
	C	G
	A	C
		T

15 (b) K-ras codon 13, position 1 - mutant (G to A, U or C)

5' - GGA GCU GGU GGC GUA GGC AAG - 3' wildtype RNA

20 5' - GGA GCU GGU HGC GUA GGC AAG - 3' mutant RNA
 3' - CCT CGA C A DCG CAT CCG TTC - 5' DNAzyme

	A	G
	G	G
25	C	C
	A	T
	A	A
	C	G
	A	C
		T

30 (c) H-ras codon 61, position 1 - mutant (C to G, U or A)

35 5' - ACC GCC GGC CAG GAG GAG - 3' wildtype RNA

5' - ACC GCC GGC DAG GAG GAG - 3' mutant RNA
 3' - TGG CGG C G HTC CTC CTC - 5' DNAzyme

	A	G
	G	G
40	C	C
	A	T
	A	A
	C	G
45	A	C
		T

(d) H-ras codon 61, position 2 - mutant (A to C, G or U)

	5' - ACC GCC GGC CAG GAG GAG - 3'	wildtype RNA
5	3' - UGG CGG CCG <u>GUC</u> CUC - 5'	<u>E</u> wildtype RNA
	5' - CUC CUG <u>CUG</u> GCC GGC GGU - 3'	<u>E</u> wildtype RNA
10	3' - GAG GA <u>GAC</u> CGG CCG CCA - 5'	DNAzyme
	A G	
	G G	
	C C	
	A T	
15	A A	
	C G	
	A C	
	T	

20 (e) H-ras codon 61, position 3 - mutant (G to C or U)

(Note; G to A is a silent mutation)

	5' - ACC GCC GGC CAG GAG GAG - 3'	wildtype RNA
25	3' - UGG CGG CCG <u>GUR</u> CUC CUC - 5'	mutant RNA antisense
	5' - CUC CUC <u>RUG</u> GCC GGC GGU - 3'	antisense
30	3' - GAG GAG <u>AC</u> CGG CCG CCA - 5'	DNAzyme
	A G	
	G G	
	C C	
35	A T	
	A A	
	C G	
	A C	
	T	

40 (f) N-ras codon 61, position 1 - mutant (C to A, G or U)

	5' - GCU GGA CAA GAA GAG - 3'	wildtype RNA
45	3' - CGA CCU HUG CUU CUC - 5'	mutant RNA <u>E</u> mutant RNA

5' - CUC UUC GUH UCC AGC - 3' E·mutant RNA
 3' - GAG AAG AD AGG TCG - 5' DNAzyme
 A G

5	G C A A C	A T A G
10	C T	A C

5' - GCU GGA UAA GAA GAG - 3' mutant RNA
 3' - CGA CC ATT CTT CTC - 5' DNAzyme

15		A	\bar{G}
		G	G
		C	C
		A	T
		A	A
20		C	G
		A	C
		T	

(2) HIV 1 - AZT Resistance, Point Mutations

25 (a) Codon 41 - mutant (A to U or C)

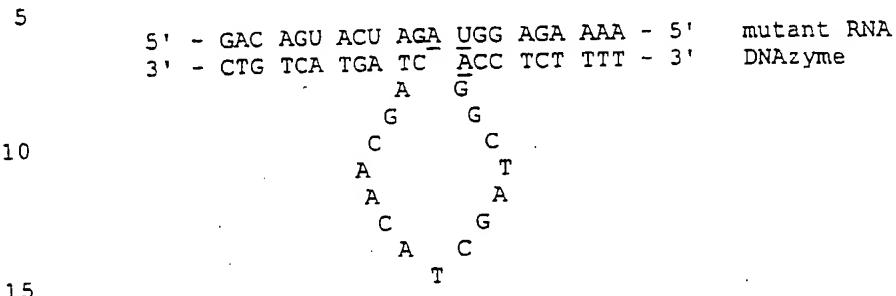
5' - UGU ACA GAA AUG GAA AAG - 3' wildtype RNA

30 5' - UGU ACA GAA YUG GAA AAG - '3' mutant RNA
 3' - ACA TGT CT RAC CTT TTC - 5' DNAzyme

35 A C T G C T A G
 G G C C T A A C G
 A A C A T T C

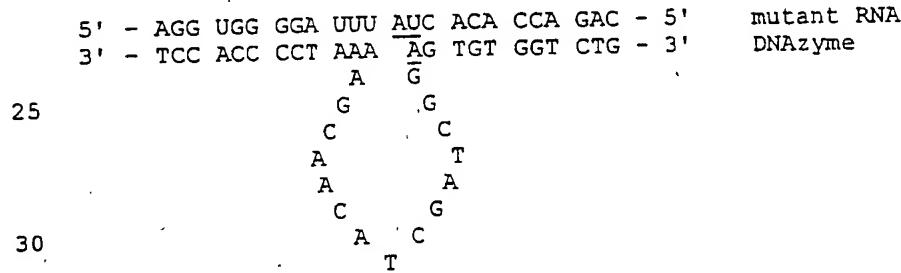
(b) Codon 70 - mutant (A to G)

5' - GAC AGU ACU AAA UGG AGA AAA - 3' wildtype
RNA

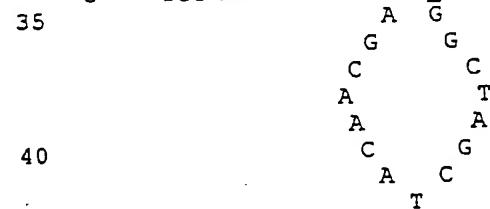


(c) Codon 215 - mutant (C to U or A)

5' - AGG UGG GGA UUU ACC ACA CCA GAC - 3' wildtype
RNA

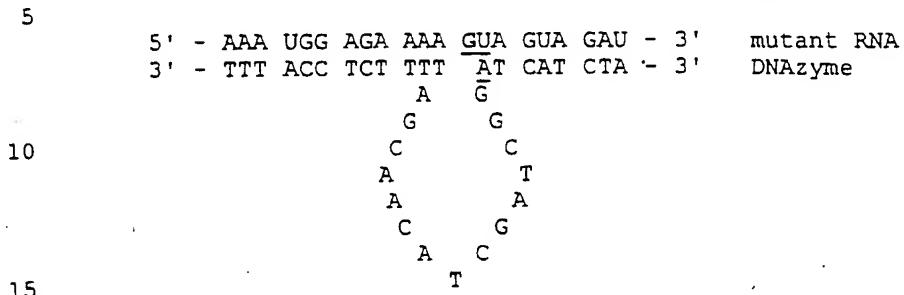


5' - AGG UGG GGA UUU AAC ACA CCA GAC - 5' mutant RNA
3' - TCC ACC CCT AAA T G TGT GGT CTG - 3' DNAzyme

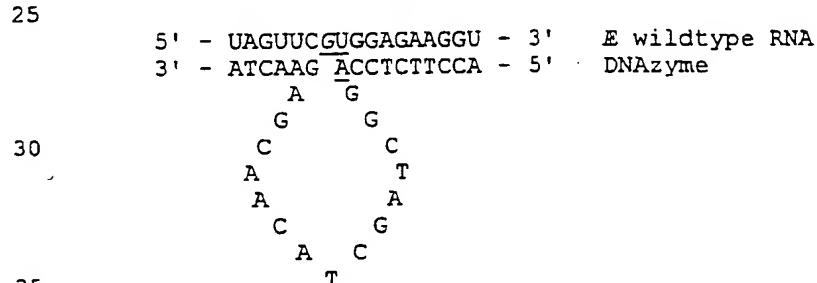


(d) Codon 74 - mutant (U to G confers ddT resistance)

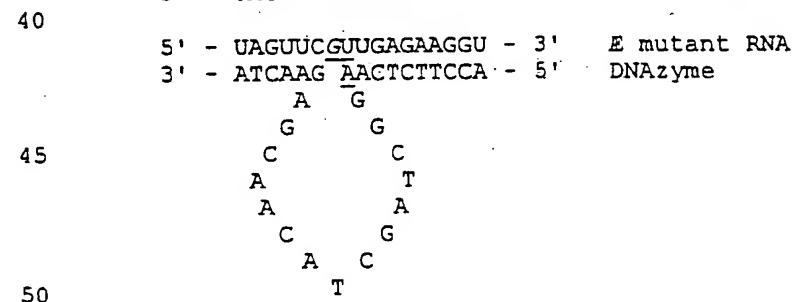
5' - AAA UGG AGA AAA UUA GUA GAU - 3' wildtype
RNA

B. Inherited Disease20 (1) Cystic Fibrosis(a) Codon 542 - wildtype

5' - UAGUUUCUUGGAGAAGGU - 3' wildtype RNA

Codon 542 - mutant (G to U)

5' - UAGUUUCUUUGAGAAGGU - 3' mutant RNA



(b) Codon 551 - wildtype

5' - GAGUGGAGGUCAACGAG - 3'
3' - CUCACCUCUCCAGUUGCUC - 5'

wildtype RNA
antisense

5
5' - CUCGUUGACCUCCACUC - 3'
3' - GAGCAAC GGAGGTGAG - 5'

antisense
DNAzyme

A G
 G
10 C C
 A T
 A A
 C G
 A C
 T
15

Codon 551 - mutant (G to A)

5' - GAGUGGAGAUCAACGAG - 3'
3' - CUCACCUCUAGUUGCUC - 5'

mutant RNA
antisense

20 5' - CUCGUUGAUCUCCACUC - 3'
 3' - GAGCAAC AGAGGTGAG - 5'

antisense
DNAzyme

A G
 G
25 C C
 A T
 A A
 C G
 A C
 T
30

(c) Codon 508 - wildtype

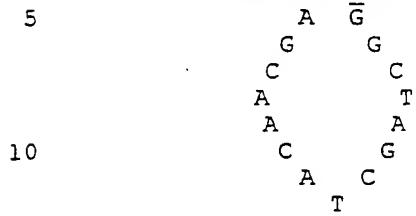
35 5' - GAAAUUAUCAUCUUUGGUGUUU - 3'
 3' - CTTTATAG AGAAACCACAAA - 5'

wildtype RNA
DNAzyme

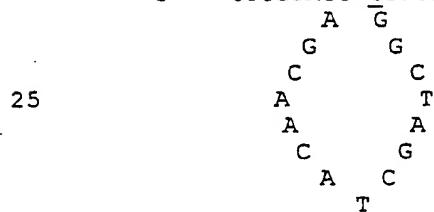
A G
 G
40 C C
 A T
 A A
 C G
 A C
 T

Codon 508 - mutant (CTT deletion)

5' - AAAUAUCAJUGGUGUUU - 5' mutant RNA
 3' - TTTATAG AACCAACAAA - 3' DNAzyme

(2) α -1-antitrypsin15 Codon 342 - mutant (G to A)

5' - GACCAUCGACGAGAAAGG - 3' wildtype RNA
 20 5' - GACCAUCGACAAGAAAGG - 3' mutant RNA
 3' - CTGGTAGC GTTCTTCC - 5' DNAzyme



II. Ribozymes

5 where bold = target base for analysis.
 underlined = recognition site (UH).

A. Acquired Disease(1) Cancer10 K-ras codon 12, position 1 - mutant (G to A, C or U)

5' - GUA GUU GGA GCU GGU GGC GUA - 3' wildtype
 RNA

15 5' - GUA GUU GGA GCU HGU GGC GUA - 3' mutant RNA
 3' - CAU CAA CCU CGA CA CCG CAU - 5' Ribozyme

A C
 A U
 G A G
 C G G A
 A U U
 G C
 G C
 25 A G
 G U

K-ras codon 12, position 2 - mutant (G to U)

30 5' - GUU GGA GCU GGU GGC GUA GGC- 3' wildtype
 RNA

35 5' - GUU GGA GCU GUU GGC GUA GGC- 3' mutant RNA
 3' - CAA CCU CGA CA CCG CAU CCG- 5' Ribozyme

A C
 A U
 G A G
 C G G A
 A U U
 40 G C
 G C
 A G
 G U

45 (2) HIV 1 - AZT resistance(a) Codon 41 - mutant (A to U or C)

50 5' - UGU ACA GAA AUG GAA AAG - 3' wildtype
 RNA

5' - UGU ACA GAA YUG GAA AAG - 3' mutant RNA
 3' - ACA UGU CUU RAC CUU UUC - 5' antisense

antisense
Ribozyme

	5'	- CUU UUC CAR UUC UGU ACA - 3'	
	3'	- GAA AAG GUY A G ACA UGU - 5'	
		A C	
		A U	
5		G A G	
		C G G A	
		A U U	
		G C	
		G C	
10		A G	
		G U	

(b) Codon 70 - mutant (A to G)

15	5'	- GAC AGU ACU AAA UGG AGA AAA - 3'	wildtype RNA
	3'	- CUG UCA UGA UCU ACC UCU UUU - 5'	
20	5'	- UUU UCU CCA UCU AGU ACU GUC - 3'	mutant RNA antisense
	3'	- AAA AGA GGU AGA CA UGA CAG - 5'	Ribozyme
		A C	
		A U	
25		G A G	
		C G G A	
		A U U	
		G C	
		G C	
30		A G	
		G U	

(c) Codon 215 - mutant (C to U or A)

35	5'	- AGG UGG GGA UUU ACC ACA CCA GAC - 3'	wildtype RNA
	3'	- UCC ACC CCU AAA UWG UGU GGU CUG - 5'	
40	5'	- AGG UGG GGA UUU AWC ACA CCA GAC - 3'	mutant RNA
	3'	- UCC ACC CCU AAA UWG UGU GGU CUG - 5'	antisense
	5'	- GUC UGG UGU GWU AAA UCC CCA CCU - 3'	antisense
	3'	- CAG ACC ACA CWA UU AGG GGU GGA - 5'	Ribozyme
		A C	
		A U	
45		G A G	
		C G G A	
		A U U	
		G C	
		G C	
50		A G	
		G U	

(d) Codon 74 - mutant. (U to G confers ddT resistance)

5' - AAA UGG AGA AAA UUA GUA GAU - 3' wildtype RNA

5' - AAA UGG AGA AAA GUA GUA GAU - 3' mutant RNA
 3' - UUU ACC UCU UUU CA CAU CUA - 5' Ribozyme
 A C
 A U
 G A G
 C G G A
 A U U
 G C
 G C
 10 A G
 G U

B. Inherited Disease

20 (1) Cystic Fibrosis

(a) Codon 542 - wildtype

25 5' - UAGUUCUJGGAGAAGGGGA - 3' wildtype
 3' - AUCAAGA CCUCUUCCACCU - 5' RNA
 30 A C
 A U
 G A G
 C G G A
 A U U
 G C
 G C
 A G
 G U
 35

Codon 542 - mutant (G to U)

mutant RNA
Ribozyme

40 5' - UAGUUCUUUGAGAAGGU - 5'
 3' - AUCAAGA ACUCUUCCCA - 3'

	A	C		
	A	U		
	G	A	G	
	C	G	G	A
45	A	U	U	
	G	C		
	G	C		
	A	G		
	G	U		

(b) Codon 551 - wildtype

5' - GAGUGGAGGUCAACGAG - 3'
 5 3' - CUCACCUCCA UUGCUC - 5'

wildtype
RNA
Ribozyme

A C
 A U
 G A G
 C G G A
 10 A U U
 G C
 G C
 A G
 G U

15 Codon 551 - mutant (G to A)

5' - GAGUGGAGGAUCAACGAG - 3'
 3' - CUCACCCUCA UUGCUC - 5'

mutant RNA
Ribozyme

A C
 A U
 G A G
 C G G A
 20 A U U
 G C
 G C
 A G
 G U

30 (c) Codon 508 - wildtype

5' - GAAAUAUCAUCUUGGUGUUU - 3'
 3' - CUUUAUAGUAGA ACCACAAA - 5'

wildtype
RNA
Ribozyme

A C
 A U
 G A G
 C G G A
 35 A U U
 G C
 G C
 A G
 G U

40 or

Codon 508 - mutant (CUU deletion)

5' - GAAAUAUCAUGGGUUU - 3'
 3' - CUUUAUAGUA CCACAAA - 5'

mutant RNA
 Ribozyme

5 A C
 A U
 G A G
 C G G A
 A U U
 10 G C
 G C
 A G
 G U

15 (2) β -Globin β + - black (poly A signal) - mutant (U to C)

20 5' - UCUGCCUAAAAAAACAU - 3'
 wildtype
 RNA

5' - UCUGCCUAACAAAAACAU - 3'
 3' - AGACGGAUUGUUUUUUGUA - 5'

25 5' - AUGUUUUUUJGUAGGCAGA - 3'
 3' - UACAAAAAAACA UCCGUCU - 5'
 antisense
 Ribozyme

30 A C
 A U
 G A G
 C G G A
 A U U
 G C
 G C
 A G
 35 G U

III. K-ras Analysis Using Ribozymes40 A. Ribozymes Targeting Mutations at K-ras Codon 12

The sequence of the human K-ras gene at codon 12 is GGT. Point mutations are frequently observed in the first 2 bases in this sequence in association with 45 cancer of the pancreas, lung and colon. Two ribozymes were designed to cleave mutant but not wild-type K-ras.

K-ras 5'.....AGUUGGAGCUHGUGGCCUAGG..... 3'
 Ribozyme I 3' UCAACCUCGA CACCGCAUCC 5'
 A C
 5 A U
 G A G
 C G G A
 A U U
 G C
 10 G C
 A G
 G U

(K-ras codon 12 - bold; Ribozyme target doublet -
 15 underlined)

Ribozyme I, above, is designed to cleave all RNA molecules which contain a point mutation at the first position of codon 12, but is designed not to cleave the wild-type sequence. The target sequence for the ribozyme is UH where H can equal C, U or A, but not G. Since the wild-type sequence is G at this position, all mutations will be cleaved with this ribozyme.

25 K-ras 5'.....UUGGAGCUGUUGGCGUAGGCA..... 3'
 Ribozyme II 3' AACCUCGACA CCGCAUCCGU 5'
 A C
 A U
 30 G A G
 C G G A
 A U U
 G C
 G C
 35 A G
 G U

(K-ras codon 12 mutant allele - bold; Ribozyme target doublet - underlined)

Ribozyme II is designed to target G to U substitutions at position 2 of codon 12. The wild-type sequence G cannot base pair with the A at the first 45 position within the hybridizing arms of the ribozyme,

and hence the wild-type sequence is not expected to be cleaved with this ribozyme.

DNA sequences encoding ribozymes I and II were
5 synthesized by Macromolecular Resources (Fort Collins,
CO). The antisense and sense strands of the ribozymes
were annealed and cloned into the vector pSP70 (Promega
Corporation, Madison, WI) behind the T7 polymerase
promoter. These clones were linearized at a site 3' to
10 the ribozyme by digestion with Nde I, then purified.
Radiolabelled ribozymes were prepared by standard *in*
vitro RNA transcription reactions which incorporated
[alpha-³²P] UTP using these templates (20).

15 B. Preparation of K-ras Templates

The human cell lines SW480 and Calu 1 were obtained
from the American Type Culture Collection (Rockville,
MD). The colon carcinoma cell line SW480 has a
20 homozygous mutation (GTT) at position 2 within codon 12
of K-ras. Calu 1 is a lung carcinoma cell line which is
heterozygous at position 1 within K-ras codon 12 having
both wild-type (GGT) and mutant (TGT) alleles.

25 K-ras DNA templates (4 mutant and 1 wild-type at
codon 12) were generated by PCR amplification of Calu 1
and SW480 DNA, as well as pUC plasmid clones which
contained K-ras inserts that were mutant at codon 12.
The sequence of the 5' PCR primer was TGGACTTAATACGA
30 CTCACTATAGGGCGACTGAATATAAAGTTGGTAG. This 5' primer
incorporated the T7 promoter at the 5' end. The
sequence of the 3' primer was CCTCTATTGTTGGATCATATTAG.
Radiolabelled K-ras RNA templates were generated by
using the T7/K-ras PCR products in standard *in vitro* RNA
35 transcription reactions which incorporated [alpha-³²P]
UTP (20).

C. Detection of Point Mutations

In vitro cleavage experiments were performed as follows. Ribozyme and substrate were incubated in cleavage buffer (10mM MgCl₂; 250mM Tris.Cl, pH 7.5) in a 4:1 molar ratio. The ribozymes I and II were incubated with the radiolabelled K-ras RNA templates at 50°C for 6hr to assess in vitro cleavage ability. Reactions were analyzed by polyacryamide gel electrophoresis. Ribozyme I successfully cleaved K-ras RNA which contained a C, A or U mutation at codon 12 position 1 but was unable to cleave the wild-type sequence G. Ribozyme II successfully cleaved K-ras RNA which contained a U mutation at codon 12 position 2 but was unable to cleave the wild-type sequence G. The presence of cleaved K-ras RNA is therefore diagnostic for the presence of point mutations at codon 12.

This analysis demonstrates the ability of catalytic nucleic acids to specifically cleave templates in vitro which provides the basis for diagnose the presence of mutant sequences associated with disease. Nucleic acid can be amplified by a variety of techniques, e.g., PCR or TMA, and then cleaved with catalytic nucleic acids, e.g., DNAzyme (10-23 model) or ribozyme. The method can be used for detection of point mutations in K-ras which are specifically associated with cancer of the lung, colon and pancreas. The approach can be applied to diagnosis of any disease which is characterized by the presence of either an acquired or inherited genetic mutation.

IV. K-ras Mutation Analysis Using 10-23 DNAzymes and Chimeric Primers

Walder, et al. (38) have previously shown that *Taq* 5 DNA polymerase can extend DNA/RNA chimeric primers that contain one or two 3' terminal ribose residues. Santoro and Joyce (19) showed cleavage of DNA/RNA chimeric substrates by the 10-23 DNAzyme. ChimERIC primers are used here to produce PCR amplicons that serve as 10 substrates for the 10-23 DNAzyme.

A. Use of DNAzymes for distinguishing variant alleles; targeting sequences with DNAzymes supplied in trans

15 Cleavage of a DNAzyme substrate produced from a chimeric primer can be achieved by adding a chemically synthesised DNAzyme to the PCR mix. In such a reaction, the DNAzyme cleaves the substrate in the *trans* 20 orientation.

(1) DNAzymes targeting mutations at K-ras codon 12; natural cleavage site

25 (a) Strategy

PCR using a 5' DNA/RNA chimeric primer (5K42r) and a 3' primer (3K2) amplified a region of the K-ras gene. 30 5K42r hybridized to the K-ras sequence adjacent to codon 12 and contained the purine:pyrimidine residues which formed the potential DNAzyme cleavage site. The chimeric primer is fully complementary to the K-ras sequence that thus provided a natural cleavage site for a 10-23 DNAzyme. Extension from the 3' end of 5K42r by *Taq* DNA 35 polymerase amplified codon 12 of the K-ras gene. A DNAzyme, Dz1, was designed to cleave amplicons that harbor wild-type sequence at codon 12 of K-ras. The 5' arm of the DNAzyme was fully complementary to sequences that are wild-type at codon 12. Mutations at K-ras

codon 12, which result in mismatches with the 5' DNAzyme-hybridizing arm, were predicted to significantly decrease the efficiency of DNAzyme cleavage.

5 (b) Primer and DNAzyme Sequences

5' TATAAAACTTGTGGTAGTTGGAGCTGGTGGCGTAGGCAA 3' K-ras
5' TATAAAACTTGTGGTAGTTGGAgcT 3' "5K42r" Primer
10 3' TGAACACCATCAACCT GACCACCG 5' "Dz1" DNAzyme
A G
G G
C C
A T
15 A A
C G
A T C

(Codon 12 in the K-ras wild-type sequence is underlined
20 and the ribonucleotide bases in the primer 5K42r are in
lower case letters.)

A further PCR primer, 3K2, was designed to produce
an 82 base-pair amplicon when amplified with 5K42r. The
25 sequence of 3K2 is:

5' CGTCCACAAAATGATTCTGA 3' "3K2" Primer

The primers and DNAzyme were synthesised by Pacific
30 Oligos Pty. Ltd. (Lismore, NSW, Australia) or Oligos
Etc., Inc. (Wilsonville, OR, USA). The DNAzyme Dz1 was
modified by adding a 3' phosphate group to prevent
extension by Taq DNA polymerase. The 5' primer, 5K42r,
was 5' end-labelled with gamma-³²P by incubating 25 ul of
35 20 uM primer with 2.5 ul of Polynucleotide Kinase (10 x
10³ U/ml, 3' phosphatase-free, Boehringer Mannheim), 2.5
ul RNasin (40 U/ml Recombinant RNasin®, Ribonuclease
Inhibitor, Promega), 5 ul of polynucleotide kinase
buffer (Boehringer Mannheim), 10 ul of gamma-³²P
40 Adenosine 5'-Triphosphate (2.5 uM, Stable Label Gold™,
Bresatec) and 5 ul of DEPC water for 30 minutes at 37°C.

(c) Preparation of K-ras Templates

pUC 18 plasmid vectors containing K-ras exon 1 sequences, which were either wild-type (GGT) or mutated at codon 12 (CGT or AGT), were used as DNA templates for PCR.

(d) Detection of Point Mutations

10 PCR mixtures contained 0.2 pg/ μ l plasmid DNA, 10 pmole of gamma- 32 P-labelled 5K42r, 2 pmole 3K2, 1 mM DTT, 8 mM MgCl₂, each dNTP (dATP, dCTP, dTTP, dGTP) at 100 μ M, 0.4 U/ μ l RNasin®, and 1 x buffer (100 mM NaCl with 50 mM Tris pH 8.3 at 25°C). Duplicate reactions were set up with 0.5 μ M Dz1, and single reactions without Dz1 were set up as control reactions. Six units of Taq DNA polymerase (5 U/ μ l AmpliTaq, Perkin-Elmer) were mixed with TaqStart™ antibody (Clontech) to give a final molar ratio of Taq DNA polymerase:TaqStart™ antibody of 1:5. The Taq DNA polymerase:TaqStart™ antibody mixture was incubated for 15 minutes at room temperature prior to addition to the PCR mix. The total reaction volumes were 50 μ l. The reactions were placed in a GeneAmp PCR 9600 (Perkin-Elmer) and denatured at 94°C for 2 minutes, then subjected to 15 cycles of 60°C for 1 minute, followed by 94°C for 20 seconds. The reaction was further subjected to 25 cycles of 40°C for 1 minute followed by 94°C for 20 seconds.

20 25 30 35 A 2.5 μ l aliquot of each reaction was mixed with 2.5 μ l of loading dye (97.5% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue and 0.01 M EDTA), incubated at 75°C for 2 minutes and then loaded immediately onto a pre-warmed 16% denaturing (urea)

acrylamide gel. The gels were electrophoresed for approximately one hour. The PCR product and cleavage fragments were visualised by scanning the gel using a Molecular Dynamics Phosphorimager 445 SI.

5

Several bands were visible on the gel (data not shown). The fragments, in order of mobility from the slowest to the fastest (i.e., from the origin to the bottom of the gel) were (a) PCR amplicons (running as a doublet), (b) unincorporated primer, and (c) cleaved PCR amplicons. Small amounts of two fragments, produced by background hydrolysis at the ribonucleotide residues within the 5' primer, were also visible running between the primer and cleaved amplicons and running parallel with the cleaved amplicons. In all reactions, PCR product and unincorporated primer were visible. Reactions containing template DNA that was wild-type at codon 12 (i.e., fully complementary to the DNAzyme) contained cleaved amplicons. Reactions containing template DNA that was mutated at codon 12 (i.e., mismatched with the DNAzyme) did not contain cleaved amplicons. Only low levels of background hydrolysis products were visible at this position on the gel in these reactions.

25

(2) DNAzymes targeting mutations at K-ras codon 12; induced cleavage site

(a) Strategy

30

PCR using a 5' DNA/RNA chimeric primer (5K44r) and a 3' primer (3K2) amplified a region of the K-ras gene. 5K44r hybridized to the K-ras sequence adjacent to codon 12 and contained the purine:pyrimidine residues which formed the potential DNAzyme cleavage site. The purine ribonucleotide in 5K44r was mismatched with respect to the K-ras template where the wild-type sequence has a

pyrimidine at this position. This primer therefore induces a DNAzyme cleavage site. Extension from the 3' end of 5K44r by Taq DNA polymerase amplified codon 12 of the K-ras gene. A DNAzyme, Dz3, was designed to cleave 5 amplicons that harbor wild-type sequence at codon 12 of K-ras. The 5' arm of the DNAzyme was fully complementary to sequences that are wild-type at codon 12. Mutations at K-ras codon 12, which result in mismatches with the 5' DNAzyme-hybridizing arm, were 10 predicted to significantly decrease the efficiency of DNAzyme cleavage.

(b) Design of PCR Primers and DNAzymes

15 5' TATAAAACTTGTGGTAGTTGGAGC3' K-ras
5' TATAAAACTTGTGGTAGTTGGAGGu 3' "5K44r" Primer
3' GAACACCATCAACCTC ACCACCGC 5' "Dz3" DNAzyme
A G
G G
20 C C
A T
A A
C G
A T C
25

(Codon 12 in the K-ras wild-type sequence is underlined, and the ribonucleotide bases in the primer 5K44r are in lower case letters. The ribonucleotide "g" in the primer 5K44r is mismatched with respect to the K-ras 30 sequence.)

A further PCR primer, 3K2, was designed to produce an 82 base-pair amplicon when amplified with 5K44r. The sequence of 3K2 is:

35 5' CGTCCACAAAATGATTCTGA 3' "3K2" Primer

The primers and DNAzyme were synthesised by Pacific Oligos Pty. Ltd. (Lismore, NSW, Australia) or Oligos 40 Etc., Inc. (Wilsonville, OR, USA). The DNAzyme Dz3 was

modified by adding a 3' phosphate group to prevent extension by Taq DNA polymerase. The 5' primer, 5K44r, was 5' end-labelled with gamma-³²P by incubating 25 ul of 20 uM primer with 2.5 ul of polynucleotide kinase (10 x 5 10³ U/ml, 3' phosphatase-free, Boehringer Mannheim), 2.5 ul RNasin (40 U/ml Recombinant RNasin®, Ribonuclease Inhibitor, Promega), 5 ul of polynucleotide kinase buffer (Boehringer Mannheim), 10 ul of gamma-³²P Adenosine 5'-Triphosphate (2.5 uM, Stable Label Gold™, 10 Bresatec) and 5 ul of DEPC water for 30 minutes at 37°C.

(c) Preparation of K-ras Templates

15 pUC 18 plasmid vectors containing K-ras exon 1 sequences, which were either wild-type (GGT) or mutated at codon 12 (CGT), were used as DNA templates for PCR.

(d) Detection of Point Mutations

20 PCR mixtures contained 0.2 pg/ul plasmid DNA, 10 pmole of gamma-³²P-labelled 5K44r, 2 pmole 3K2, 1 mM DTT, 8 mM MgCl₂, each dNTP (dATP, dCTP, dTTP, dGTP) at 100 uM, 0.4 U/ul RNasin®, and 1 x buffer (100 mM NaCl with 50 mM Tris pH 8.3 at 25°C). Duplicate reactions were set up 25 with 0.5 uM Dz3 DNazyme, and single reactions without Dz3 were set up as control reactions. Six units of Taq DNA polymerase (5 U/ml AmpliTaq, Perkin-Elmer) were mixed with TaqStart™ antibody (Clontech) to give a final molar ratio of Taq DNA polymerase:TaqStart™ antibody of 30 1:5. The Taq DNA polymerase:TaqStart™ antibody mixture was incubated for 15 minutes at room temperature prior to addition to the PCR mix. The total reaction volumes were 50 ul. The reactions were placed in a GeneAmp PCR 9600 (Perkin-Elmer) and denatured at 94°C for 2 minutes, 35 then subjected to 30 cycles of 60°C for 1 minute,

followed by 94°C for 20 seconds. The reaction was further subjected to 10 cycles of 50°C for 1 minute followed by 94°C for 20 seconds.

5 A 2.5 ul aliquot of each reaction was mixed with 2.5 ul of loading dye (97.5% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue and 0.01 M EDTA), incubated at 75°C for 2 minutes and then loaded immediately onto a pre-warmed 16% denaturing (urea) 10 acrylamide gel. The gels were electrophoresed for approximately 1 hour. The PCR product and cleavage fragments were visualised by scanning the gel using a Molecular Dynamics Phosphorimager 445 SI.

15 Several bands were visible on the gel (data not shown). The fragments, in order of mobility from the slowest to the fastest (i.e., from the origin to the bottom of the gel) were (a) PCR amplicons (running as a doublet), (b) unincorporated primer, and (c) cleaved PCR 20 amplicons. Small amounts of a fragment, produced by background hydrolysis at the ribonucleotide bond within the 5' primer, was also visible running parallel with the cleaved amplicons. In all reactions, PCR product and unincorporated primer were visible. Reactions containing 25 template DNA that was wild-type at codon 12 (i.e., fully complementary to the DNAzyme) contained cleaved amplicons. Reactions containing template DNA that was mutated at codon 12 (i.e., mismatched with the DNAzyme) did not contain cleaved amplicons. Only low levels of background 30 hydrolysis products were visible at this position on the gel in these reactions.

B. DNAzymes targeting mutations at K-ras codon 12; cleavage in cis orientation

Cleavage of amplicons produced from a chimeric primer can also be achieved using active DNAzymes that are synthesised during PCR. In one example of such a reaction the DNAzyme is cleaving the substrate in the *cis* orientation.

10 (a) Strategy

PCR using a 5' DNA/RNA chimeric primer (5K42r) and a 3' zymogene primer (3K42Dz2) amplified a region of the K-ras gene. 5K42r hybridized to the K-ras sequence adjacent to codon 12 and contained the purine:pyrimidine residues which formed the potential DNAzyme cleavage site. The zymogene primer 3K42Dz2 had a 3' region that was complementary to K-ras, and a 5' region that contained the antisense of a DNAzyme. The zymogene primer had no inherent catalytic activity itself but, when used in conjunction with 5K42r, it facilitated the production of amplicons which had a DNAzyme cleavage site near their 5' termini and active (sense) DNAzymes at their 3' termini. The DNAzyme is designed to cleave the 5' end of the amplicons *in cis*. The 5' arm of the DNAzyme was fully complementary to sequences that are wild-type at codon 12. Mutations at K-ras codon 12, which result in mismatches with the 5' DNAzyme arm, were predicted to significantly decrease the efficiency of DNAzyme cleavage.

(b) Primer Sequences

35 5' chimeric primer 5K42r
(upper case - deoxyribonucleotide residues; lower case - ribonucleotide residues)

5' TATAAAACTTGTGGTAGTTGGAgcT 3'

3' zymogene primer 3K42Dz2
5 complement (antisense) of 10:23 catalytic core in
bold)

5' ACTTGTGGTAGTTGGATCGTTGTAGCTAGCCCTGG
TGGCAGCTGTATCGTCAAGGCACTC 3'

10 The primers were synthesised by Pacific Oligos Pty.
Ltd. (Lismore, NSW, Australia) or Oligos Etc., Inc.
(Wilsonville, OR, USA). The 5' primer, 5K42r, was 5'
end-labelled with gamma-³²P by incubating 25 ul of 20 uM
15 primer with 2.5 ul of polynucleotide kinase (10×10^3
U/ml, 3' phosphatase-free, Boehringer Mannheim), 2.5 ul
RNasin (40 U/ml Recombinant RNasin®, Ribonuclease
Inhibitor, Promega), 5 ul of polynucleotide kinase
buffer (Boehringer Mannheim), 10 ul of gamma-³²P
20 Adenosine 5'-Triphosphate (2.5 uM, Stable Label Gold™,
Bresatec) and 5 ul of DEPC water for 30 minutes at 37°C.

(c) K-ras DNA Templates

25 pUC 18 plasmid vectors containing K-ras exon 1
sequences, which were either wild-type (GGT) or mutated
at codon 12 (CGT or AGT), were used as DNA templates for
PCR.

30 (d) Cleavage in cis by DNAzymes synthesised during the
PCR

PCR mixtures contained 0.2 pg/ul K-ras plasmid DNA,
10 pmole of gamma-³²P-labelled 5K42r, 2 pmole 3K42Dz2, 1
35 mM DTT, 8 mM MgCl₂, each dNTP (dATP, dCTP, dTTP, dGTP) at
100 uM, 0.4 U/ul RNasin®, and 1 x buffer (100 mM NaCl

with 50 mM Tris pH 8.3 at 25°C). Duplicate reactions were set up for each DNA template. Six units of Taq DNA polymerase (5 U/ul AmpliTaq, Perkin-Elmer) were mixed with TaqStart™ antibody (Clontech) to give a final molar ratio of Taq DNA polymerase:TaqStart™ antibody of 1:5. The Taq DNA polymerase:TaqStart™ antibody mixture was incubated for 15 minutes at room temperature prior to addition to the PCR mix. The total reaction volumes were 50 ul. The reactions were placed in a GeneAmp PCR 9600 (Perkin-Elmer), denatured at 94°C for 2 minutes, subjected to 30 cycles at 60°C for 1 minute, followed by treatment at 94°C for 20 seconds. The reaction was further subjected to 10 cycles at 50°C for 1 minute, followed by treatment at 94°C for 20 seconds.

A 2.5 ul aliquot of each reaction was mixed with 2.5 ul of loading dye (97.5% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue and 0.01 M EDTA), incubated at 75°C for 2 minutes, and then loaded immediately onto a pre-warmed 16% denaturing (urea) acrylamide gel. The gels were electrophoresed for approximately 1 hour. The PCR product and cleavage fragments were visualized by scanning the gel using a Molecular Dynamics Phosphorimager 445 S1.

Several bands were visible on the gel (data not shown). The fragments, in order of mobility from the slowest to the fastest (i.e., from the origin to the bottom of the gel) were (a) PCR amplicons (running as a doublet), (b) unincorporated primer, and (c) cleaved PCR amplicons. Small amounts of two fragments, produced by background hydrolysis at the ribonucleotide residues within the 5' primer, were also visible running between the primer and cleaved amplicons and running parallel with the cleaved amplicons. In all reactions, PCR

product and unincorporated primer were visible. Reactions containing template DNA that was wild-type at codon 12 (i.e., fully complementary to the DNAzyme) contained cleaved amplicons. Reactions containing 5 template DNA that was mutated at codon 12 (i.e., mismatched with the DNAzyme) did not contain cleaved amplicons. Only low levels of background hydrolysis products were visible at this position on the gel in these reactions.

10

The sequence below is an amplicon that is wild-type at position 1 of codon 12 (underlined) shown in a conformation wherein the DNAzyme (**bold**) is hybridizing in *cis*.

15

5' TATAAAACTTGTGGTAGTTGGAgc**TGGTGGCGTAGGCAAGAGTGC**
3' TGAACACC**ATCAACCT GACCACCGTCGACATAGCAGTT**
A G
20 G G
C C
A T
A A
C G
25 A T C

30

V. Conclusion
The instant diagnostic methods are advantageous. Catalytic nucleic acids can require as few as two base pairs of specific sequence to create a cleavage site. Catalytic nucleic acid dinucleotide cleavage sites occur 35 naturally at a greater frequency than do restriction enzyme cleavage sites. Furthermore, mismatched primers can be used to induce cleavage sites for catalytic nucleic acids in the same way that mismatched primers have been used to induce artificial restriction enzyme 40 cleavage sites.

Examples of catalytic nucleic acids which require only a dinucleotide sequence at the cleavage site are the hammerhead ribozyme and the 10-23 DNAzyme. Both 5 these molecules also require complementarity between the hybridizing regions (arms) and the molecule to be cleaved. However these regions can be made target-specific. Although catalytic nucleic acid molecules can only cleave single-stranded nucleic acid templates, 10 methods of generating suitable single-stranded templates are well known in the art. For example, single-stranded RNA templates can be generated by a protocol such as TMA, and single-stranded DNA can be generated by asymmetric PCR (37) or by the denaturation of double- 15 stranded products.

The instant methods provide a new tool for sequence analysis that is potentially more flexible than analysis by RFLP. The combination of nucleic acid amplification 20 with catalytic nucleic acid cleavage overcomes the limitations of analysis using restriction enzymes. Here, the minimum sequence requirement for cleavage has been reduced. Furthermore, since the catalytic nucleic acid must also be complementary in the hybridizing 25 region, these regions which flank the dinucleotide cleavage site will also effect cleavage efficiency. The length of sequence scanned by one catalytic nucleic acid can therefore be greater than that scanned by a single restriction enzyme. The analysis of sequences using 30 catalytic nucleic acids also has an advantage over other protocols since here, no protein enzymes (e.g., restriction enzymes or RNAase A) or toxic compounds are required.

References

1. Mullis, K.B., U.S. Patent No. 4,683,202.
- 5 2. Arnheim, N., et al., U.S. Patent No. 4,683,195.
3. Arnheim, N., et al., U.S. Patent No. 4,000,159.
- 10 4. Ehrlich H.A., et al., U.S. Patent No. 4,965,188.
5. Ehrlich H.A., et al., U.S. Patent No. 5,176,995.
6. F.F. Chehab, et al. (1987) *Nature* 329:293-294.
- 15 7. R.K. Saiki, et al. (1985) *Science* 230:1350-1354.
8. Barany, F. (1991) *Proc. Natl. Acad. Sci.* 88:189-193.
- 20 9. Walker, G.T., et al. (1992) *Nucleic Acids Res.* 20:1691.
10. Jonas, V., et al. (1993) *Journal of Clinical Microbiology* 31:2410-2416.
- 25 11. Fahy, E., et al. (1991) *PCR Methods Appl* 1: 25-33.
12. Haseloff, J. and Gerlach, W.L. (1988) *Nature* 334:585-591.
- 30 13. Breaker, R.R. and Joyce, G. (1994) *Chemistry and Biology* 1:223-229.
- 35 14. Koizumi, M., et al. (1989) *Nucleic Acids Research* 17:7059-7069.

15. E. Otsuka and M. Koizumi, Japanese Patent No. 4,235,919.
- 5 16. Kashani-Sabet, M., et al. (1992) *Antisense Research and Development* 2:3-15.
- 10 17. Raillard, S.A. and Joyce, G.F. (1996) *Biochemistry* 35:11693-11701.
18. Carmi, N., et al. (1996) *Chemistry and Biology* 3:1039-1046.
- 15 19. Santoro, S.W. and Joyce, G. (1997) *PNAS* 94:4262-4266.
20. 20. *Promega Protocols and Applications Guide*. Titus, D.E. (Ed), Promega Corporation (1991).
- 20 21. Watson, J.D., Tooze, J. and Kurtz, D.T. (1983) *Recombinant DNA: A short Course*. Scientific American Books, New York.
- 25 22. Antonarakis, S.E. (1989) *New England Journal of Medicine* 320:153-163.
23. Perriman, R. and Gerlach, W.L. (1992) *Gene* 113:157-163.
- 30 24. Nollau-Wagener, P. (1997) *Clinical Chemistry* 43: 1114-1128.
- 25 25. Carmi, N., et al. (1996) *Chemistry and Biology* 3:1039-1046.

26. Kramvis, A., et al. (1996) *Journal of Clinical Microbiology* 34: 2731-2733.
27. Yong, S.L., Thomas, R.J.S. and Phillips, W.A. (1995) *Nucleic Acids Research* 23:1640.
28. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., New York: Cold Spring Harbour Laboratory Press.
29. Backus, J.W., et al., U.S. Patent No. 5,582,988.
30. Wei, L., Dai-Shu, H., Ju, Y. and Andrieu, J.-M. (1994) *Nature* 368; 269-271.
31. Bradley, S.M., et al., PCT International Publication No. WO 84/01389.
32. Cohen, J.B. and Levinson, A.D. (1988) *Nature* 334:119-124.
33. Kumar, R. and Barbacid, M. (1988) *Oncogene* 3:647-651.
34. Todd, A.V., et al. (1991) *Leukemia* 5:160.
35. Levi, S., et al. (1991) *Cancer Res.* 6:1079.
36. Kwok, S., et al. (1990) *Nucleic Acids Research* 18:999-1005.
37. Gyllensten, U.B. and Erlich, H.A. (1988) *PNAS* 95:7652-7656.
38. Walder, R.Y., et al. (1993) *Nucleic Acid Research* 21(18):4339-4343.

What is claimed is:

1. A method of determining whether a subject is afflicted with a disorder characterized by the presence of a known nucleic acid mutation, which comprises the steps of
 - (a) isolating a sample of nucleic acid molecules from the subject;
 - (b) (i) amplifying the nucleic acid segment present in the isolated sample, which segment is known to contain the mutation in a subject afflicted with the disorder, and (ii) under suitable conditions, contacting the resulting amplified segment with a catalytic nucleic acid molecule which specifically recognizes and cleaves a target sequence present either (1) in the nucleic acid segment having the known mutation or (2) in the corresponding wild-type nucleic acid segment, but not both, with the proviso that step (ii) can be performed either subsequent to or concurrently with step (i); and
 - (c) determining whether the catalytic nucleic acid molecule in step (b) (ii) cleaves the amplified segment, so as to determine whether the subject is afflicted with the disorder.
2. A method of determining whether a subject is afflicted with a disorder characterized by the presence of a plurality of known nucleic acid mutations, which comprises the steps of
 - (a) isolating a sample of nucleic acid molecules from the subject;
 - (b) (i) amplifying the nucleic acid segment present in the isolated sample, which segment is known to contain the plurality of mutations in a subject afflicted with the disorder, and (ii)

- 5
- 10
- 15
- 20
- 25
- 30
- 35
- under suitable conditions, contacting the resulting amplified segment with a plurality of catalytic nucleic acid molecules, each of which specifically recognizes and cleaves a target sequence present either (1) in the nucleic acid segment having the known mutation or (2) in the corresponding wild-type nucleic acid segment, but not both, with the proviso that step (ii) can be performed either subsequent to or concurrently with step (i); and
- (c) determining whether each of the catalytic nucleic acid molecules in step (b) (ii) cleaves the amplified segment, so as to determine whether the subject is afflicted with the disorder.
3. A method of determining whether a subject is afflicted with a disorder characterized by the presence of a plurality of known nucleic acid mutations, which comprises the steps of
- (a) isolating a sample of nucleic acid molecules from the subject;
- (b) (i) amplifying the nucleic acid segments present in the isolated sample, which segments collectively are known to contain the plurality of mutations in a subject afflicted with the disorder, and (ii) under suitable conditions, contacting the resulting amplified segments with a plurality of catalytic nucleic acid molecules, each of which specifically recognizes and cleaves a target sequence present either (1) in one of the nucleic acid segments having one of the known mutations or (2) in the corresponding wild-type nucleic acid segment, but not both, with the proviso that step (ii) can be performed

- either subsequent to or concurrently with step
(i); and
- (c) determining whether each of the catalytic
nucleic acid molecules in step (b)(ii) cleaves
the amplified segment containing its respective
target sequence, so as to determine whether the
subject is afflicted with the disorder.
- 5
4. The method of claim 1, 2 or 3, wherein the subject is
10 a human.
5. The method of claim 1, 2 or 3, wherein the disorder
is selected from the group consisting of cancer, AIDS
and cystic fibrosis.
- 15
6. The method of claim 5, wherein the disorder is
cancer.
7. The method of claim 1, 2 or 3, wherein the amplified
20 nucleic acid segment is RNA and the catalytic nucleic
acid molecule is selected from the group consisting
of DNA and RNA.
8. The method of claim 1, 2 or 3, wherein the amplified
25 nucleic acid segment is DNA and the catalytic nucleic
acid molecule is RNA or DNA.
9. The method of claim 1, 2 or 3, wherein (a) the
amplification is performed using a polymerase chain
30 reaction; (b) the catalytic nucleic acid molecule is
a 10-23 DNAzyme; and (c) the polymerase chain
reaction employs a DNA primer suitable for initiating
amplification of the segment, which primer contains
at least one purine ribonucleotide residue which
35 serves as the 5' side of the site within the

amplified segment recognized and cleaved by the 10-23 DNAzyme.

10. The method of claim 9, wherein the amplified segment
5 is recognized and cleaved *in trans* by the DNAzyme.

11. The method of claim 9, wherein (a) the polymerase chain reaction employs a second DNA primer suitable for initiating amplification of the segment, which
10 second primer comprises a zymogene encoding a 10-23 DNAzyme such that, upon amplification, the resulting amplified nucleic acid molecule comprises the 10-23 DNAzyme; and (b) the amplified nucleic acid segment is recognized and cleaved *in cis* by the DNAzyme.
15

12. A kit for use in practicing the method of claim 1,
2 or 3, which comprises (a) a catalytic nucleic acid molecule which specifically recognizes and cleaves a target sequence present either (i) in a
20 nucleic acid segment having a mutation known to be characteristic of a disorder or (ii) in the corresponding wild-type nucleic acid segment, but not both, and (b) a nucleic acid reagent suitable for use in amplifying the nucleic acid segment containing
25 the target sequence.

13. A kit for use in practicing the method of claim 9,
which comprises (a) a 10-23 DNAzyme which specifically recognizes and cleaves a target
30 sequence present either (i) in a nucleic acid segment having a mutation known to be characteristic of a disorder or (ii) in the corresponding wild-type nucleic acid segment, but not both, and (b) a DNA primer suitable for initiating amplification of the
35 segment under polymerase chain reaction conditions, which primer contains at least one purine

ribonucleotide residue which serves as the 5' side of the site within the amplified segment recognized and cleaved by the 10-23 DNAzyme.

5 14. A kit for use in practicing the method of claim 11, which comprises

(a) a first DNA primer which comprises a zymogene encoding a 10-23 DNAzyme that specifically recognizes and cleaves a target sequence present either (i) in a nucleic acid segment having a mutation known to be characteristic of a disorder or (ii) in the corresponding wild-type nucleic acid segment, but not both, which first primer is suitable for initiating amplification of the segment under polymerase chain reaction conditions; and

10 (b) a second DNA primer suitable for initiating amplification of the segment under polymerase chain reaction conditions, which second primer contains at least one purine ribonucleotide residue which serves as the 5' side of the site within the amplified segment recognized and cleaved by the 10-23 DNAzyme, such that, upon amplification, (i) the resulting amplified nucleic acid molecule comprises the 10-23 DNAzyme, and (ii) the amplified nucleic acid segment is recognized and cleaved in *cis* by the DNAzyme.

15
20
25
30

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB 99/00848

A. CLASSIFICATION OF SUBJECT MATTER		
Int Cl ⁶ : C12Q 1/68		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched AU: IPC C12Q 1/68		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) WPAT, MEDLINE, CA, BIOT; KEYWORDS: DNA ENZYME#, RNA ENZYME#, RIBOZYME#, DNAZYME#, ZYMOGENE#, PCR, AMPLIFICATION, LIGASE CHAIN REACTION, MUTATION#, POLYMORPHISM, ALLELE#		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Nature Biotechnology Vol. 17 No.1 pages 62-66 issued January 1999. Robertson MP et al, "In vitro selection of an allosteric ribozyme that transduces analytes to amplicons." Whole article	1-14
Y	AU 20718/92 A (AKZO NV) 11 March 1993 Claims 6, 7	1-14
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C		<input checked="" type="checkbox"/> See patent family annex
<ul style="list-style-type: none"> * Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed 		<ul style="list-style-type: none"> "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family
Date of the actual completion of the international search 12 July 1999	Date of mailing of the international search report 28 JUL 1999	
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929	Authorized officer ROSS OSBORNE Telephone No.: (02) 6283 2404	

INTERNATIONAL SEARCH REPORT

International application No. PCT/IB 99/00848
--

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 95/19448 A (JOHN HOPKINS SCHOOL OF MEDICINE) 20 July 1995 claims	1-14
P, A	Biochem Biophys Res Commun Vol. 249, No. 3, pages 804-810, issued 28 August 1998 Phylactou LA et al "Hammerhead ribozymes targeted to the FBN1 mRNA can discriminate a single base mismatch between ribozyme and target"	
P,A	WO 99/09162 A (US SECRETARY OF HEALTH & HUMAN SERVICES) 25 February 1999	

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/IB 99/00848

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report				Patent Family Member			
AU	20718/92	CA	2075147	EP	525882	FI	922447
		JP	5219999	US	5834255	ZA	9205631
WO	9519448	AU	16038/95	CA	2181147	EP	749495
WO	9909162	AU	90162/98				

END OF ANNEX